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A COINCIDENCE TECHNIQUE FOR PAPER CHROMATOGRAPHY OF SOME BIOLOGICALLY IMPORTANT COMPOUNDS

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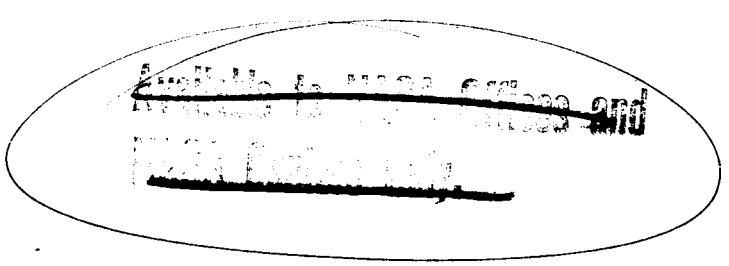

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

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A COINCIDENCE TECHNIQUE FOR PAPER CHROMATOGRAPHY OF SOME BIOLOGICALLY IMPORTANT COMPOUNDS

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Abstract

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A method has been developed for the unambiguous identification of some biologically important compounds by paper chromatography. Shadowgrams on photographic paper and autoradiographs on X-ray film have been combined to give a single precise technique. The uncertainty which arises from the identification based on R_f values alone is eliminated.

AUTHOR

Introduction

The isolation and identification of trace quantities of a host of natural and synthetic substances has been made possible by the use of paper chromatography. The R_f value or ratio of distance traveled by substance to the distance traveled by solvent front, is generally used as the criterion of identity. If two substances have the same R_f values in two or more solvent systems, they are assumed to be identical. But although one often sees in the literature, R_f values quoted to an astonishing degree of precision, experience shows that R_f values are at best only a rough guide, and should not be used as the sole means of identifying a substance.

R_f values are dependent on a large number of variables, among which may be mentioned:

1. Composition of development phase
2. Kind of paper
3. Direction of paper
4. Manner of development (descending, ascending, ascending-descending, radial)
5. Length of paper used for development
6. Distance of starting line from solvent
7. Concentration of solute
8. Presence of other substrates
9. Temperature of development

The uncertainty accompanying the identification by R_f values alone is illustrated very pertinently from the following study from some of our work on the radiolysis of C^{14} labeled adenine.[1] An autoradiograph of an

irradiated aqueous solution of adenine is shown on the right of figure 1. Two major products are indicated, A and B. We identified A as 4-6 diamino 5-formamido pyrimidine and B as 8-hydroxyadenine. The chromatographic identification was further confirmed by chemical tests and ultraviolet spectrophotometry.

At the same time, however, another group of workers reported that compound B was adenine 1-N-oxide, basing their identification on R_f values alone.[2] The following R_f values for 8-hydroxyadenine and adenine 1-N-oxide indicated that no precise differentiation could have been made on this criterion alone:

	Adenine-8-OH	Adenine-1-N-oxide
Propanol-ammonia-water	0.47	0.51
Butanol-propionic acid	.61	.66
Isobutyric acid-ammonia	.54	.58
Butanol-water	.62	.64

To establish the identity of compound B the chromatography of the original sample was repeated, adding adenine-1-N-oxide as a carrier. The solvent systems were composed of propanol-ammonia-water and butanol-propionic acid-water. The bright areas on the ultraviolet absorption picture (shadowgram) on the left of figure 1 are the adenine-1-N-oxide and the residual adenine. The dark spot B on the autoradiograph appears to be almost coincident with the bright area on the shadowgram. Superimposition of the autoradiograph on the shadowgram indicated that coincidence was not altogether precise. When the experiment was then repeated in two other solvent systems, isobutyric acid-ammonia and butanol-water, the spot produced

by the unlabeled adenine-1-N-oxide shifted further away from the radioactive spot B (figure 2). If B was indeed adenine-1-N-oxide, the radioactivity and the absorption would have coincided in both systems as in the case of the residual adenine.

In our experimental work we have developed a technique which gives unambiguous results. Ultraviolet absorption photography has been used as early as 1949 by Markham and his co-workers for the location of purines and pyrimidines.[8] Autoradiography has been extensively used since the pioneering work of Calvin and his associates with C^{14}.[4] In the work we are reporting, the two techniques have been combined as one operation. This method has been successfully used for purines, pyrimidines, amino acids and sugars.

Experimental

Compounds labeled C^{14} are used as starting materials in all reactions studied. Nonradioactive carriers of the suspected resultant products of the experiment are added to the original material before chromatography. The mixture is then chromatographed in two dimensions on Whatman no. 4 paper, previously washed with oxalic acid.

Autoradiographs are prepared by placing an X-ray film in close contact with the chromatogram for a period determined by the amount of radioactive material present. This time may range from one day to six months. We have found that 10,000 dpm or 5 micromicrocuries produces a fairly well defined spot on the X-ray film in approximately a week. Radioactive compounds appear as dark spots on the transparent X-ray film. If the radioactive

products are identical with the carriers, the dark spots on the autoradiograph will correspond precisely to the bright spots on the shadowgram.

When working with purines, pyrimidines, and other ultraviolet absorbing products, the shadowgrams are prepared by shining an ultraviolet light through the chromatogram on to Kodagraph contact paper. A General Electric germicidal lamp was used as the ultraviolet source from a distance of ten inches. The time of exposure is 1-2 seconds. To insure close contact of the papers and therefore sharper prints, a sheet of vycor glass which transmits light of the wavelength at which purines and pyrimidines absorb is placed between the light and the paper. The ultraviolet absorbing areas on the chromatogram appear as well-defined white spots on a dark background.

For greater certainty, the area corresponding to the matching dark and bright spots is cut out from the paper chromatogram, eluted with a suitable solvent, and rerun in two different solvent systems. This is the equivalent of analysis in four separate systems. If coincidence is established with this repeated procedure, the positive identification of the material can be assumed.

With amino acids or sugars, the carriers are located on the chromatogram with the aid of a color reaction. The shadowgram is prepared in the same manner as for the purines and pyrimidines, but a visible light source is used instead of an ultraviolet light. The colored areas absorb the light and a well-defined bright spot appears on a dark background. Figures 3, 4, and 5 demonstrate the results obtained with purines and pyrimidines, amino acids and sugars. In all these cases, it is understood that the amount of radioactive material is insufficient to be detected by a shadowgram alone.

This identification technique can be performed in reverse order also. When starting with unlabled material, a radioactive carrier is added before spotting. The amount of radioactive product should be below the limit of detectability for the shadowgram but large enough to produce a dark spot on the X-ray film. The limit of detectability when using unlabeled material is about 0.003 μg for purines and pyrimidines, 0.04 μg for amino acids and about 1 μg for the sugars.

Discussion

Our work has been primarily concerned with purines, pyrimidines, amino acids and sugars. This coincidence technique, however, can be applied to many other compounds which will give a color reaction such as fatty acids, peptides, steroids, etc., in fact, to any compound which can absorb ultraviolet or visible light. This technique was used for the successful identification of adenine among a large number of compounds formed by the electron irradiation of a mixture of methane, ammonia and water.[5] In further experimental work on primordial organic synthesis the same method was instrumental in the identification of adenosine triphosphate.[6] The application of this coincidence technique provides an unambiguous identification of compounds without the uncertainty involved in using R_f values and often eliminating the need for tedious chemical analysis.

Acknowledgments

This work was begun when one of us (Cyril Ponnamperuma) was a member of the Bio-Organic Chemistry Group, Lawrence Radiation Laboratory, Berkeley 4, California. He thanks Dr. Richard M. Lemmon and Professor Melvin Calvin for advice, help and encouragement.

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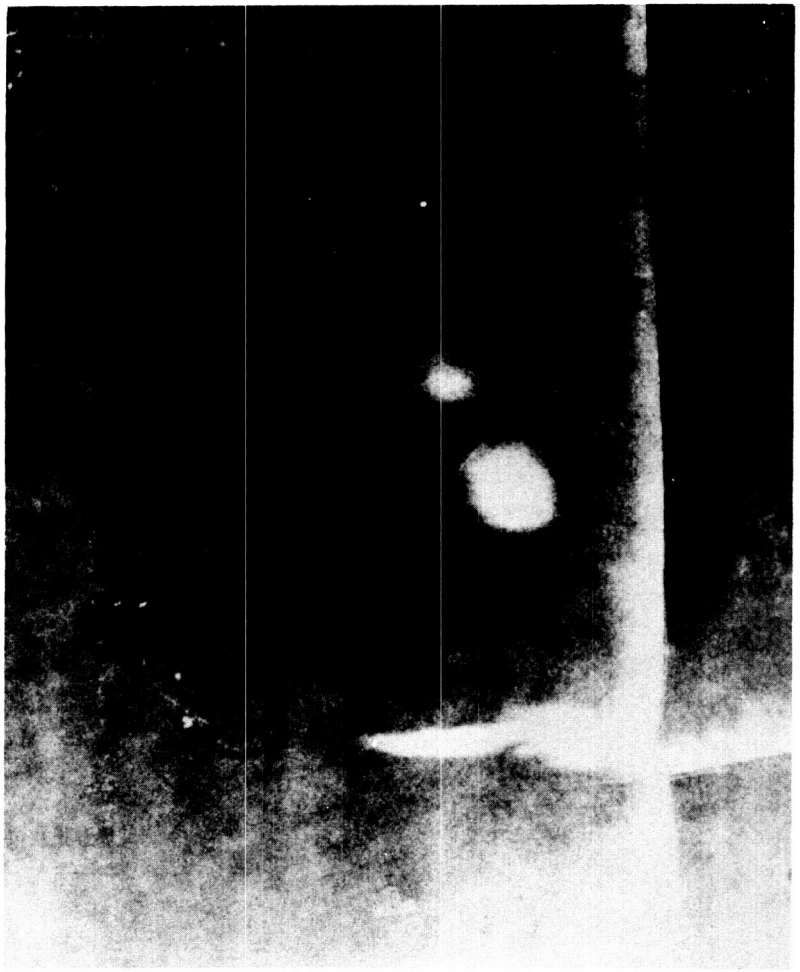

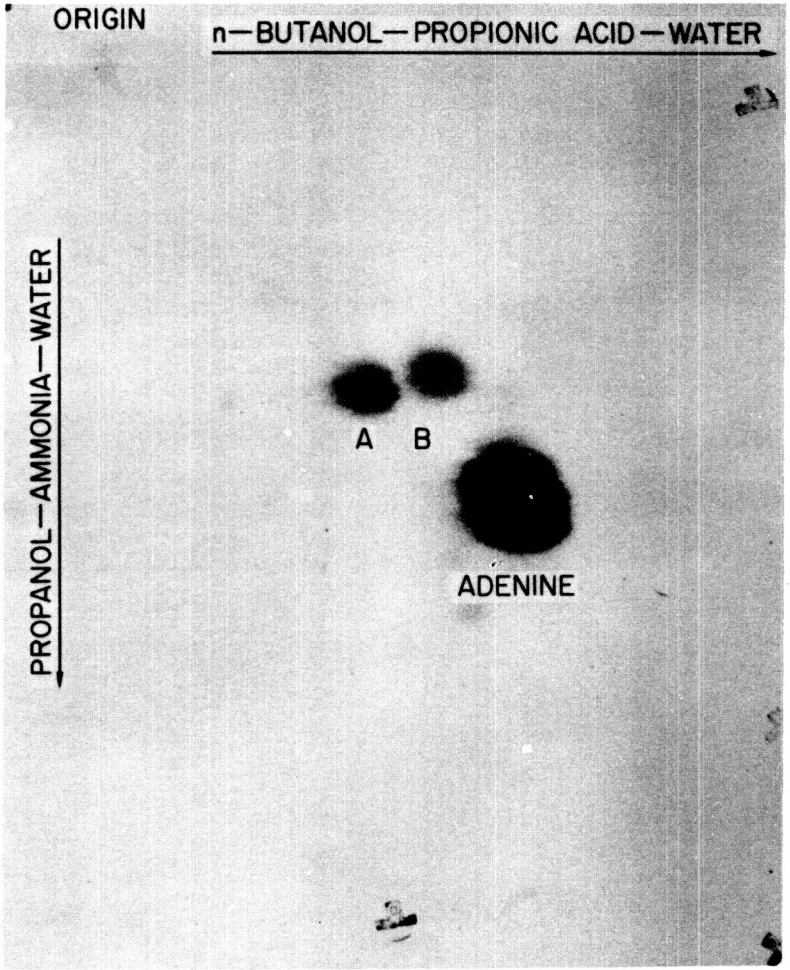

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Figure 1.- Autoradiograph shows the radiolysis of Adenine. Shadowgram shows carrier Adenine. The solvents are n-Butanol-Propionic Acid-Water - Propanol-Ammonia-Water. There appears to be coincidence between B and the small white spot on the Shadowgram.

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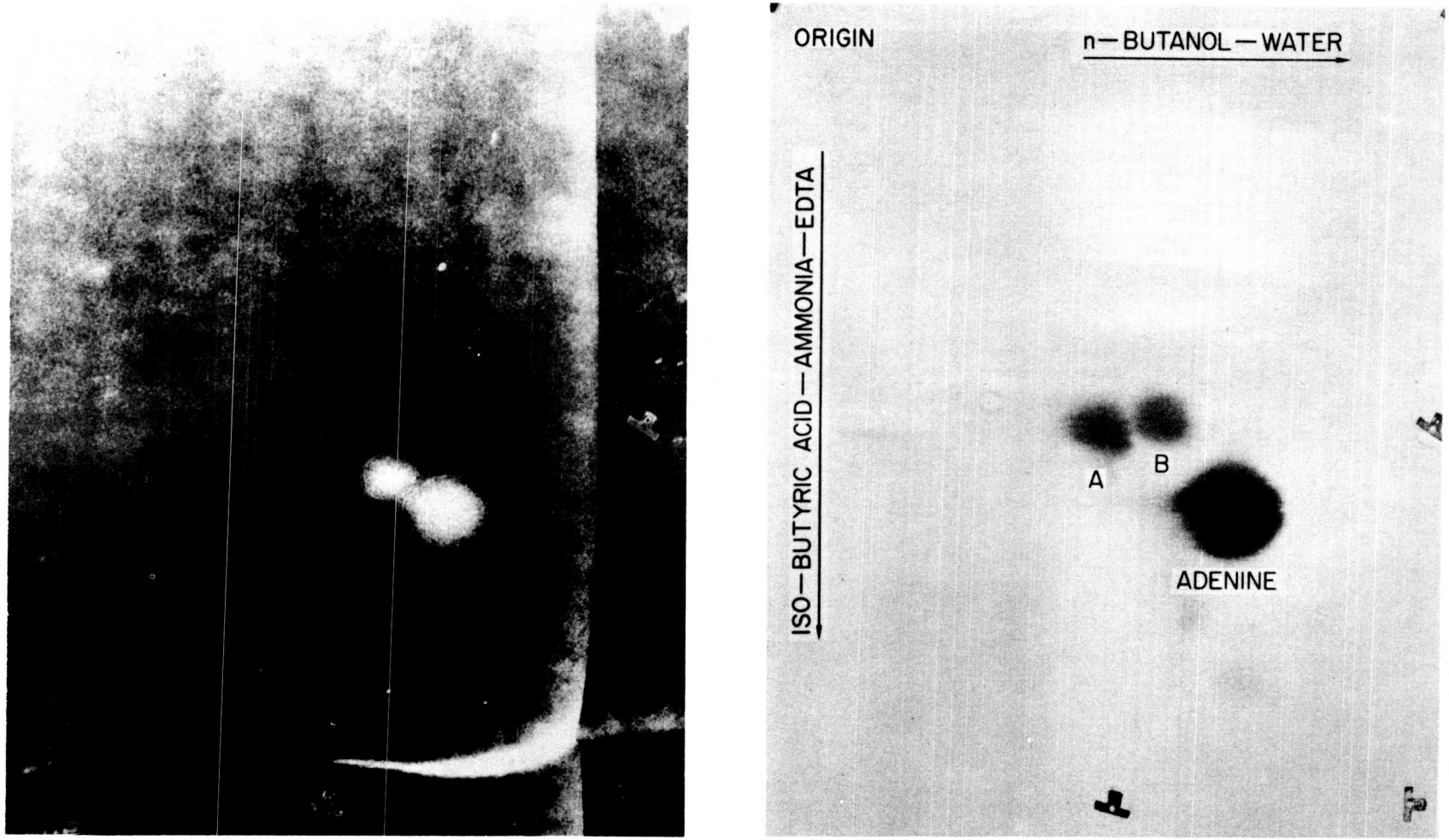

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Figure 2.- Autoradiograph shows the radiolysis products of Adenine. Shadowgram shows carrier Adenine-1-N-oxide and undecomposed Adenine. The solvents are n-Butanol-water and Iso-Butynic Acid-Ammonia-EDTA. Note that the small white spot on the Shadowgram no longer appears to coincide with B.

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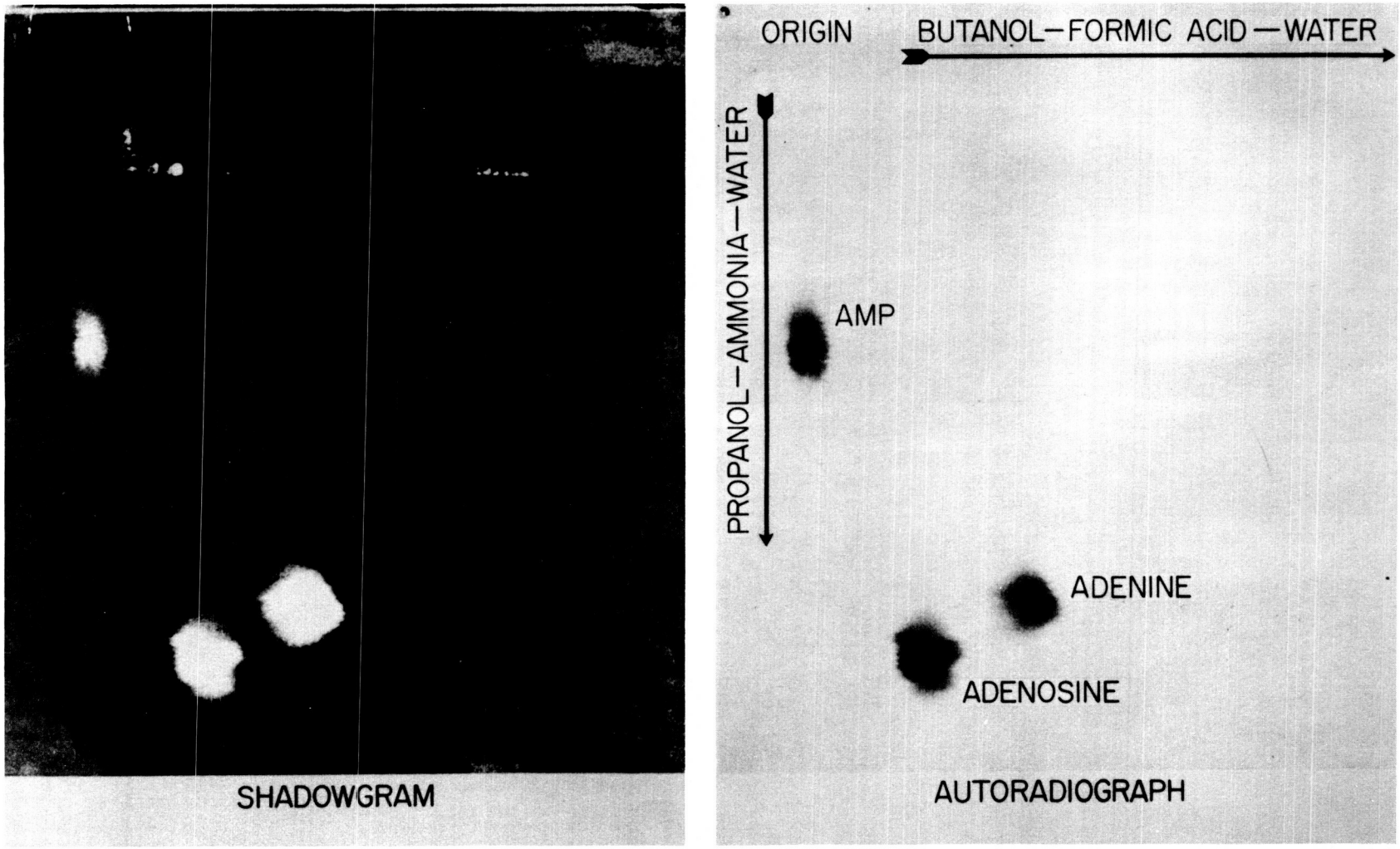

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Figure 3.- Identification of UV absorbing compounds.

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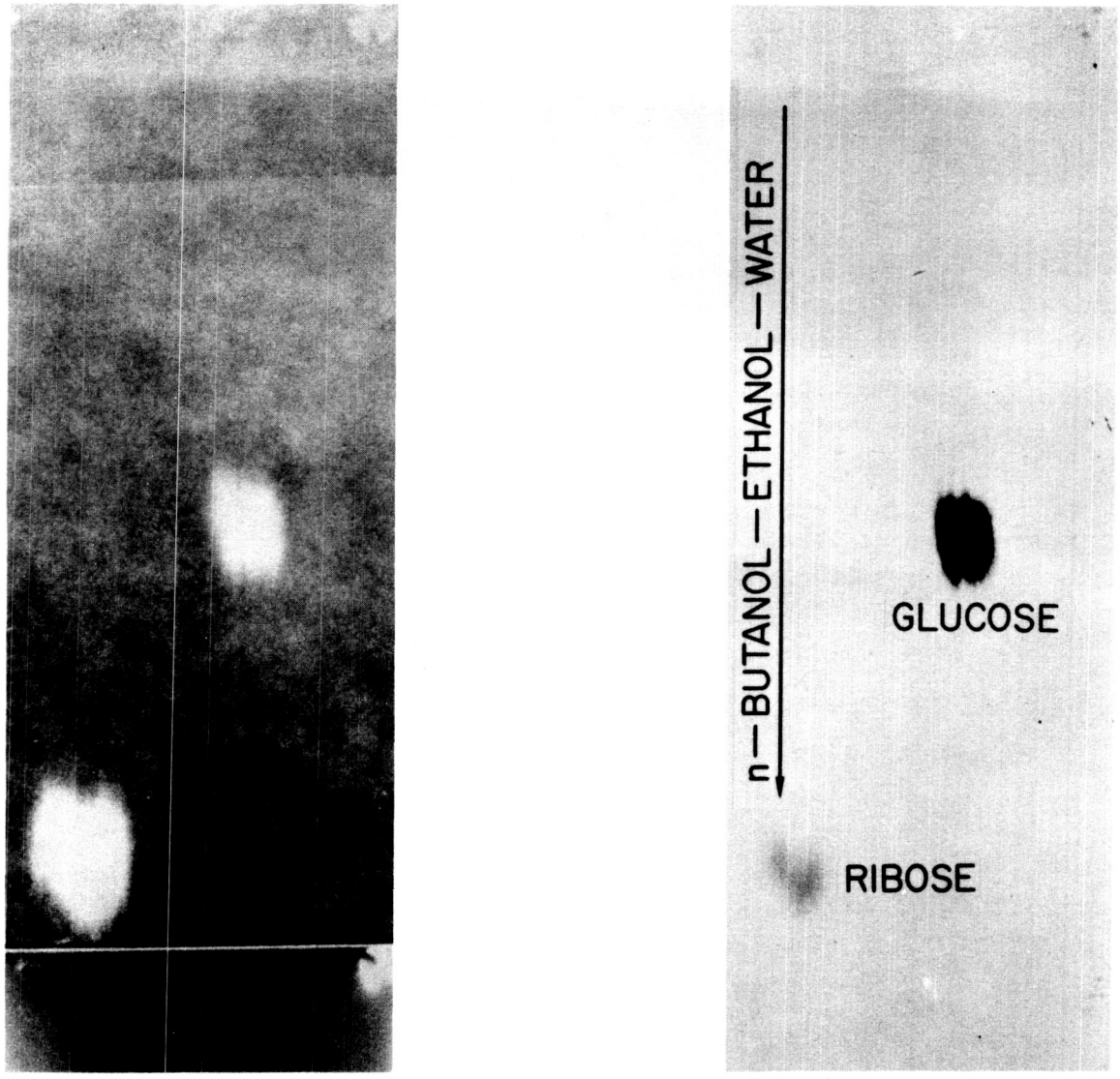

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Figure 4.- Identification of sugars.

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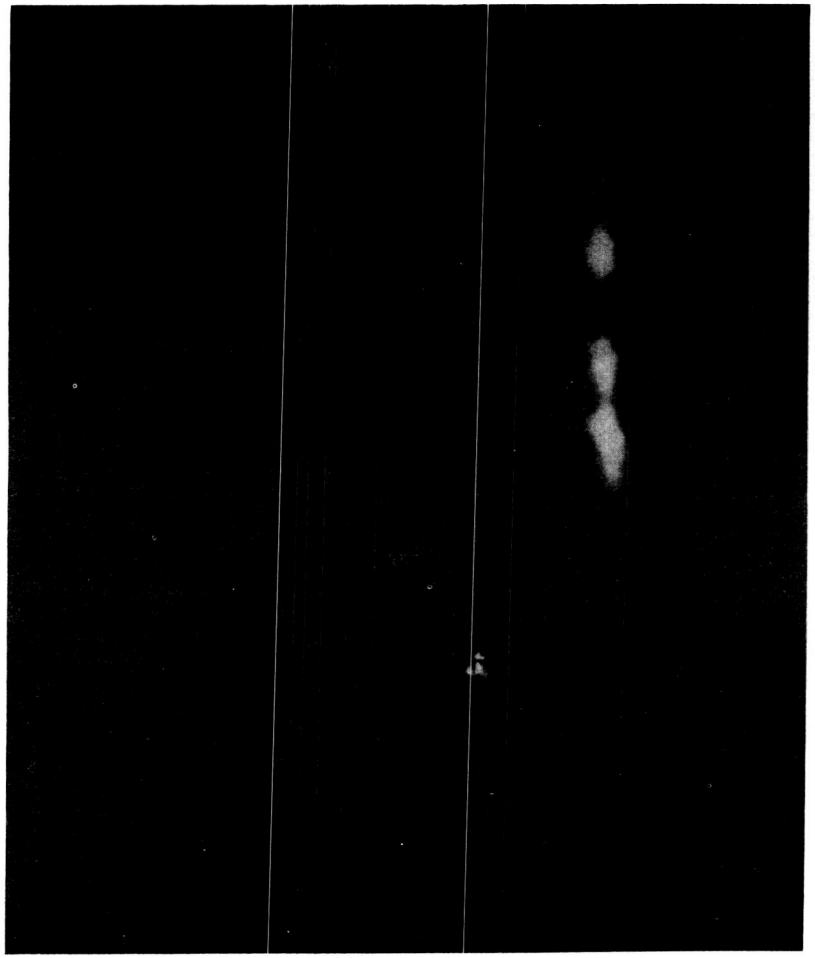

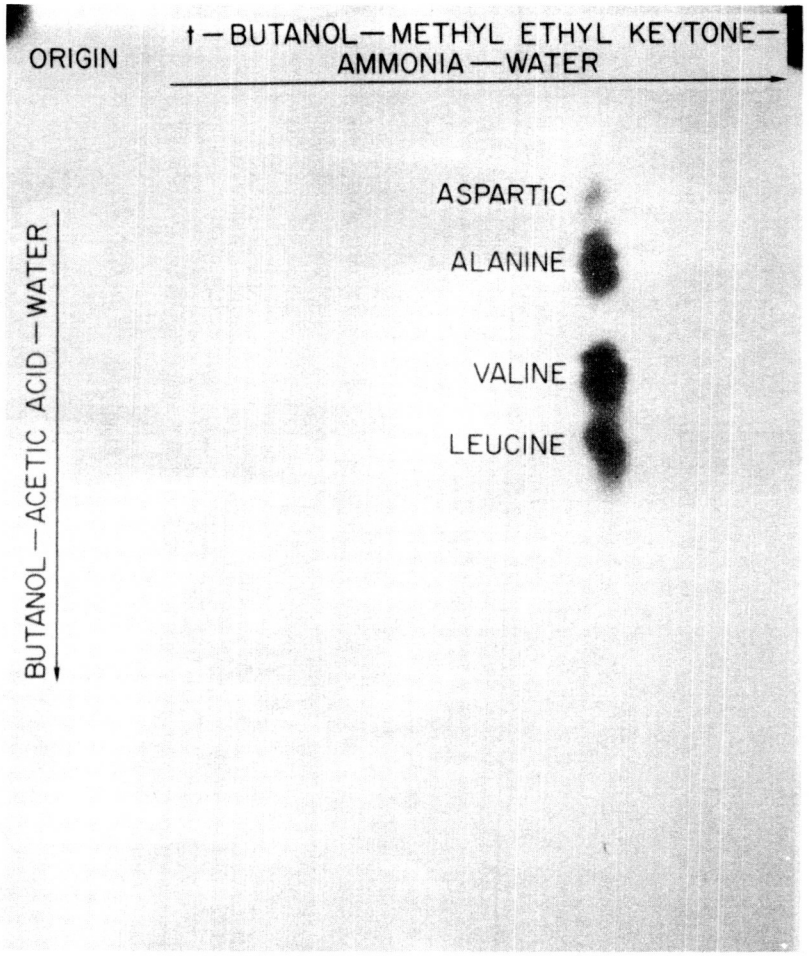

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Figure 5.- Identification of Amino Acids.

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